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DNA coding for ß-tubulin and its use

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The invention relates to DNA which codes for β-tubulin from nematodes of the family of the Strongylidae, the polypeptide encoded by this DNA, the use of the DNA for the diagnosis of anthelmintic resistance of these nematodes and for the identification of the species of these nematodes, the use of the β-tubulin as a constituent of a vaccine, and a process for the identification of new anthelmintic or antibiotic compounds.

Parasitic helminths (worms) are a health problem for humans and animals and cause significant economic damage. In 1996, the expenditure for anthelmintics worldwide was more than 2 billion US dollars. The most important anthelmintics which are presently used can be divided into three groups according to their mechanism of action:

1. The cyclic amidines pyrantel and morantel act, together with the imidazothiazoles tetramisole and levamisole, as cholinergic compounds for the parasitic nervous system.

2. The benzimidazoles are inhibitors of the polymerization of microtubules and lead to the degradation of tubulin, followed by the loss of a number of cell functions such as transport within cells and cell division.

3. The macrocyclic lactones bind and open glutaminergic chloride channels and thus act as inhibitors of the nervous system of nematodes and arthropods.

Microtubules are built up from tubulin subunits. Tubulin is a dimeric protein which consists of α- and ß-tubulin and is in a dynamic equilibrium between tubulin and microtubules. This equilibrium can be influenced by exogenous substances, which are designated as microtubule inhibitors. Some of these inhibitors, such as, for example,

the benzimidazoles, act by their binding to tubulin, whereby they prevent the self-association of these subunits to growing microtubules, while at the opposite end the dissociation of the microtubules is continued. On account of this, dysfunctions occur in processes within the cell which are important to life and finally the death of the cell and of the entire organism occurs (Lacey, E. (1990) Mode of action of benzimidazoles. Parasitology Today 6, 112-115). Such microtubule inhibitors include various classes of compound which are synthetically prepared or produced by various organisms.

The binding of microtubule inhibitors to tubulin from various organisms shows great differences with respect to the affinity of the binding. Thus the anthelmintics oxfendazole and thiabendazole show a high affinity for tubulin from *Ascaridia galli* and an only slight affinity for tubulin from mammals such as the sheep (Dawson et al. (1983) Purification and characterisation of tubulin from the parasitic nematode, *Ascaridia galli*, Molecular and Biochemical Parasitology 7, 267-277). The selective toxicity of benzimidazoles can be explained by means of this selective affinity (Lacey, E. (1988) The role of the cytoskeletal protein, tubulin, in the mode of action and mechanism of drug resistance to benzimidazoles, International Journal for Parasitology 18, 885-936).

The widespread use of these anthelmintics has led to considerable resistance problems against all three classes, especially in livestock (Bauer et al. (1994) Anthelmintic resistance in nematodes of farm animals. A seminar organised for the European Commission, Brussels, Belgium, 8 to 9 November 1993, pp. 17-24). The most widespread class of anthelmintics are the benzimidazoles. Resistance to benzimidazoles has been described worldwide in the case of parasites of sheep, cattle, pigs and horses. Benzimidazoles are broad-spectrum anthelmintics with action against nematodes, cestodes and trematodes. Investigations in German stud farms showed a resistance of small strongylids to benzimidazoles in more than 80% of the cases (Ullrich et al. (1988) Benzimidazole resistance in small strongylids

(Cyathostominae): distribution in horse stock in Northrhine-Westphalia, Berliner Münchner Tierärztliche Wochenschrift 101, 406-408).

For an effective treatment with anthelmintics, it is therefore of great importance to obtain information about possible resistance of worms in a horse or within a herd of horses. In order to check the possible resistance of a worm population of small stronglids, diagnostic procedures have already been developed which are based on the activity of anthelmintics in the stages of development of the parasites (eggs, larvae) (Coles et al. (1992) World Association for the Advancement of Veterinary Parasitology (W.A.A.V.P.) Methods for the detection of anthelmintic resistance in nematodes of veterinary importance, Veterinary Parasitology, 44, 35-44). The "larval development assay" (LDA) describes the inhibitory effect of anthelmintics in the nonparasitic first larval stage as a function of the anthelmintic concentration employed. In a similar manner, the second approach, the "egg hatch assay" (EHA) uses the inhibitory action of the anthelmintics on the hatching of the larvae as a function of the anthelmintic concentration employed. A disadvantage of both approaches lies in the low reproducibility of the results. Moreover, both approaches are time-consuming and labor-intensive.

The sensitivity of both approaches is moreover low. An existing resistance is only detected if more than 25% of the population are resistant (Roos et al. (1995) New genetic and practical implications of selection for anthelmintic resistance in parasitic nematodes, Parasitology Today 11, 148-150).

There is therefore an urgent need for diagnostic procedures which are sensitive, rapid and reproducible. For the sheep parasites, *Haemonchus contortus* and *Teladorsagia circumcincta,* a procedure based on the PCR technique has been described. This is based on at least one point mutation in codon 200 of the ß-tubulin isotype 1 gene (Elard et al. (1999) PCR diagnosis of benzimidazole - susceptibility or - resistance in natural populations of the small ruminant parasite, *Teladorsagia circumcincta*, Veterinary Parasitology 80, 231-237). The point mutations in codon 200 result in an

amino acid replacement of phenylalanine by tyrosine and correlates with a benzimidazole resistance of the mutated protein (Kwa et al. (1995) ß-tubulin genes from parasitic nematode *Haemonchus contortus* modulate drug resistance in *Caenorhabditis elegans*, Journal of Molecular Biology 246, 500-510).

From studies on *Haemonchus contortus,* it is known that the sequences of nematodes coding for tubulin are species-specific (WO 92/03549).

The various species of the small strongylids of the horse differ in their epidemiological frequency. The species which are most important and found most frequently worldwide include *Cylicocyclus nassatus*, *Cyathostomum coronatum* and *Cyathostomum catinatum*. Resistances of these species to benzimidazoles have been described in various countries, inter alia also in Germany (Burger, H.-J. and Bauer, C. (1987) Efficacy of four anthelmintics against benzimidazole - resistant cyathostomes of horses, Veterinary Record 120, 293-296).

The nucleic acid sequencing of ß-tubulin cDNAs from the species of small strongylids according to the invention had an identity of over 95%. The identity with the known, abovementioned ß-tubulin sequences of sheep parasites, however, is only 75.4-82.6%.

The derived amino acid sequences are very similar within the sequences according to the invention. This is also true of the derived ß-tubulin amino acid sequences of the sheep parasites. The identity here is between 95 and 99.8%. Only very few positions result in which amino acid exchange occurs. To be emphasized here is codon 200, in which a change from phenylalanine to tyrosine results.

However, the noncoding ß-tubulin sequences from various helminth species published hitherto show no significant identity. It is accordingly surprising that not only the coding sequences, but also parts of the noncoding sequences, of the various species of small strongylids of the application present here have a high identity.

These regions are therefore also suitable to be able to differentiate various species of small strongylids and other nematode species from one another. PCR primers which are derived from these intron regions can be used for the specific detection of small strongylids within a sample which also contains genetic material from other helminth organisms.

ß-Tubulin from nematodes or parts thereof are known for having a protective, immunological potential (Bughio et al. (1993) Characterisation and biological activities of anti-*Brugia pahangi* tubulin monoclonal antibodies, International Journal for Parasitology, 7, 913-924). The ß-tubulin of the small strongylids encoded by the abovementioned DNA can be used as a vaccine just as monoclonal antibodies can be used against the ß-tubulin.

Inhibitors of the interaction of the tubulin or of its subunits, such as benzimidazoles, colchizine and taxol, are important lead structures of a series of therapeutics which are directed against human, animal or plant diseases. The importance of tubulin as the target of these compounds is far-reaching and underlines its potential for the search for new active compounds for the control of these diseases.

The invention relates to the DNA coding for the ß-tubulin from nematodes of the family of the Strongylidae, particularly of the subfamily of the Cyathostominae, or fragments of this DNA. The ß-tubulin DNA can in this case be genomic DNA or cDNA. The DNA sequences which this invention relates to can be considered as new members of the tubulin gene family of parasitic nematodes of the order Strongylida, particularly of the subfamily of the Cyathostominae. The invention relates very particularly to the DNA sequences which code for ß-tubulin from parasitic nematodes of the genera Cyathostomum and Cylicocyclus.

The invention likewise relates to DNA sequences which have an identity of more than 85% to a polynucleotide coding for one of the amino acid sequences as set forth in SEQ ID NO. 2, 4, 6, 8 or 10.

The invention likewise relates to preferred DNA sequences which have an identity of more than 95% to a polynucleotide coding for one of the amino acid sequences as set forth in SEQ ID NO. 2, 4, 6, 8 or 10.

The invention relates in particular to DNA sequences coding for ß-tubulin, which originate from parasitic nematodes of the genera Cylicocyclus and Cyathostomum, very particularly those sequences which originate from parasitic nematodes of the species *Cylicocyclus nassatus*, preferably DNA as set forth in SEQ ID NO. 3, 5, 7, 9 or 11 or from *Cyathostonum coronatum*, preferably DNA according to SEQ ID NO. 1.

The invention likewise relates to DNA sequences as described above, which in contrast to these sequences have at least one point mutation or one nucleotide replacement in codon 200. These point mutations result in a change in the amino acid sequence encoded by this DNA, e.g. a replacement of the amino acid phenylalanine by tyrosine, and correlate with the resistance of tubulin with appropriate mutations to benzimidazoles.

The invention likewise relates to DNA sequences which are complementary to the DNA described above or fragments of this DNA, and to fragments of these DNA sequences. These DNA sequences or these fragments comprise oligonucleotides which are derived from one of the DNA sequences mentioned above or described under SEQ ID NO. 1, 3, 5, 7, 9 or 11 or sequences identical to 85% thereto, preferably sequences identical to 95%, and are derived from strands complementary thereto and can hybridize to these.

The invention in this case relates preferably to oligonucleotides consisting of or comprising one of the sequences as set forth in SEQ ID NO. 12 to SEQ ID NO. 51, which hybridize to abovementioned DNA sequences, preferably in the region of noncoding sequence sections of the ß-tubulin genes.

The invention likewise preferably relates to oligonucleotides consisting of or comprising one of the sequences as set forth in SEQ ID NO. 12 to SEQ ID NO. 51, which hybridize to coding regions of the abovementioned sequences.

The invention likewise relates to RNA sequences which are complementary to the DNA described above or fragments of this DNA, and fragments of these RNA sequences. These RNA sequences or these fragments comprise ribooligonucleotides which correspond to a region of one of the DNA sequences mentioned above or described under SEQ ID NO. 1, 3, 5, 7, 9 or 11, sequences complementary thereto or DNA sequences 85% identical, preferably 95% identical, thereto, and can hybridize to these.

The invention likewise relates to an expression construct which comprises one of the DNA sequences described above, and to a DNA sequence linked thereto which makes possible the expression of the DNA. These include, for example, at least one promoter for constitutive or inducible expression or alternatively enhancers. Suitable promoters for expression in *E. coli* are natural hybrid or bacteriophage promoters, preferably promoters of the group of λ-phages, hsp, omp or synthetic promoters such as mentioned, for example, in WO 98/5625, DE 3 430 683 or EP 0 173 149.

The invention likewise relates to vectors which comprise one of the DNA sequences described above and make possible the expression of the ß-tubulin according to the invention or fragments thereof in a host cell.

The invention likewise relates to host cells which contain the abovementioned DNA, an expression construct as mentioned above, or a vector and allow the expression of the ß-tubulin or fragments thereof.

The invention likewise relates to polypeptides which are encoded by one of the abovementioned DNA sequences or fragments of these DNA sequences, and to fragments of these polypeptides.

The invention in this case relates preferably to polypeptides which are encoded by a DNA sequence comprising SEQ ID NO. 1, 3, 5, 7, 9 or 11, by DNA sequences which have an identity of 85% to these sequences, preferably of 95%, or of fragments of this DNA.

The invention also relates to polypeptides which are encoded by the DNA sequence described above, which contain at least one point mutation in codon 200 as described above and show resistance to benzimidazoles, and to fragments of these polypeptides.

The invention in this case relates very particularly preferably to polypeptides comprising one of the amino acid sequences described in SEQ ID NO. 2, 4, 6, 8 or 10 or fragments thereof.

The invention in this case relates to polypeptides, particularly to purified polypeptides or polypeptides prepared recombinantly.

The invention relates to polypeptides of full length and also to corresponding fragments of these polypeptides, e.g. certain motifs or domains. These fragments can be of different length and comprise, for example, 5, 10, 25, 50, 100, 150, 200, 250 or 300 amino acids.

This invention likewise relates to fusion proteins which comprise a polypeptide as described above. The fusion protein can in this case contain a further polypeptide component which is not connected to the ß-tubulin (e.g. LexA, B42, glutathione S-transferase, a His tag, a polypeptide having enzymatic activity such as alkaline phosphatase or an epitope tag).

The invention also relates to a process for the preparation of a polypeptide as described above in suitable prokaryotic or eukaryotic expression systems. The expression can in this case be carried out permanently or transiently as described above in a corresponding cell line in each case or corresponding host cells. Suitable prokaryotic expression systems are known host-vector systems such as bacteria (e.g. *Streptomyces spp., Bacillus subtilis, Salmonella typhimurium, Serratia marcescens* and particularly *Escherichia coli*).

Expression in a eukaryotic system is preferably carried out in the baculovirus system, particularly in a system which allows the introduction of post-translational modifications.

This invention likewise relates to the use of DNA as mentioned above for the detection of DNA from nematodes of the family Strongylidae, preferably of the subfamily Cyathostominae, particularly preferably of the genera Cyathostomum and Cylicocyclus, very particularly preferably of the species *Cyathostomum coronatum* and *Cylicocyclus nassatus*. The invention in this case relates to oligonucleotides as mentioned above which are complementary to DNA coding for ß-tubulin or strands complementary thereto and can hybridize to this DNA. Preferably, these oligonucleotides hybridize to the intron regions, i.e. the noncoding DNA sequences. The invention relates to the use of these oligonucleotides or parts thereof as

a) samples in Northern or Southern blot assays,

b) PCR primers in a diagnostic procedure for the detection of the abovementioned nematodes, the DNA of the nematodes concerned being specifically identified and amplified with the aid of the primer and the PCR technique.

The invention in this case relates preferably to oligonucleotides consisting of or comprising one of the sequences as set forth in SEQ ID NO. 12 to SEQ ID NO. 51.

The invention likewise relates to the use of DNA as mentioned above for the detection of DNA from nematodes of the family of the Strongylidae, preferably of the subfamily of the Cyathostominae, particularly preferably of the genera Cylicocyclus and Cyathostomum, very particularly preferably of the species *Cylicocyclus nassatus* and *Cyathostomum coronatum*, which codes for ß-tubulin or fragments thereof, which are resistant to benzimidazoles. The invention in this case relates to oligonucleotides as mentioned above which are complementary to DNAs which code for ß-tubulin with a resistance to benzimidazoles or to the complementary strands of this DNA and which can specifically hybridize to this DNA.

The invention also relates to the use of these oligonucleotides or parts thereof as

a) samples in Northern or Southern blot assays,

b) PCR primers in a diagnostic procedure for the detection of the abovementioned nematodes with a resistance to benzimidazoles, the DNA of the nematodes concerned being specifically identified and amplified with the aid of the primer and the PCR technique.

The invention in this case preferably relates to oligonucleotides consisting of or comprising one of the sequences as set forth in SEQ ID NO. 12 to SEQ ID NO. 51.

The invention also relates to a procedure for the detection of nematodes of the family of the Strongylidae, preferably of the subfamily of the Cyathostominae, particularly preferably of the genera Cylicocyclus and Cyathostomum, very particularly preferably of the species *Cylicocyclus nassatus* and *Cyathostomum coronatum*, where oligonucleotides as described above specifically hybridize to DNA sequences which originate from the organisms mentioned, and which can be amplified with the aid of

the PCR technique. The hybridization is preferably carried out in the noncoding regions of the β-tubulin gene (introns).

The detection of organisms as mentioned above can be carried out, for example, by

a) making available an oligonucleotide probe or primer which can hybridize to the abovementioned DNA coding for ß-tubulin or strands complementary thereto or to the 5'- or 3'-flanking regions thereof,

b) bringing the oligonucleotide probe or the primers into contact with an appropriately prepared probe containing DNA,

c) detecting the hybridization of the oligonucleotide or primer (e.g. with the aid of the polymerase chain reaction),

d) sequencing the detected sequence of the ß-tubulin gene, and

e) comparing this sequence with the DNA sequences according to the invention which were described above, preferably with DNA sequences as set forth in SEQ ID NO. 1, 3, 5, 7, 9 or 11.

The invention also relates to a process for the detection of nematodes of the family of the Strongylidae, preferably of the subfamily of the Cyathostominae, particularly preferably of the genera Cylicocyclus and Cyathostomum, very particularly preferably of the species *Cylicocyclus nassatus* and *Cyathostomum coronatum*, which are resistant to benzimidazoles, where oligonucleotides as described above specifically hybridize to DNA sequences which originate from the organisms mentioned and which can be amplified with the aid of the PCR technique. The hybridization is preferably carried out in the noncoding regions of the β-tubulin gene (introns).

The detection of organisms as mentioned above can be carried out, for example, by

a) making available an oligonucleotide probe or primer which can hybridize to the abovementioned DNA coding for ß-tubulin or strands complementary thereto or to the 5'- or 3'-flanking regions thereof,

b) bringing the oligonucleotide probe or the primers into contact with an appropriately prepared probe containing DNA,

c) detecting the hybridization of the oligonucleotide or primer (e.g. with the aid of the polymerase chain reaction),

d) sequencing the detected sequence of the ß-tubulin gene, and

e) comparing this sequence with the DNA sequences according to the invention which were described above, preferably with DNA sequences as set forth in SEQ ID NO. 1, 3, 5, 7, 9 or 11, having at least one point mutation in codon 200, which leads to a resistance of the β-tubulin encoded by these sequences to benzimidazoles.

The invention likewise relates to a diagnostic test kit for the detection and identification of nematodes of the family of the Strongylidae, preferably of the subfamily of the Cyathostominae, particularly preferably of the genera Cylicocyclus and Cyathostomum, very particularly preferably of the species *Cylicocyclus nassatus* and *Cyathostomum coronatum*, which, inter alia, makes available oligonucleotides as described above, which can be used in procedures for the detection of the species mentioned. The present invention likewise makes available oligonucleotides which specifically hybridize to sequences as set forth in SEQ ID NO. 1, 3, 5, 7, 9 or 11, sequences complementary thereto, sequences having at least one point mutation in codon 200, or fragments of these sequences. In this case, oligonucleotides consisting

of or including sequences as set forth in SEQ ID NO. 12 to SEQ ID NO. 51 are particularly preferred.

The invention likewise relates to a diagnostic test kit for the detection of nematodes of the family of the Strongylidae, preferably of the subfamily of the Cyathostominae, particularly preferably of the genera Cylicocyclus and Cyathostomum, very particularly preferably of the species *Cylicocyclus nassatus* and *Cyathostomum coronatum*, having a resistance to benzimidazoles which, inter alia, makes available oligonucleotides as described above, which can be used in procedures for the detection of the species mentioned. The present invention likewise makes available oligonucleotides which hybridize specifically to sequences as set forth in SEQ ID NO. 1, 3, 5, 7, 9 or 11, sequences complementary thereto, sequences having at least one point mutation in codon 200, or fragments of these sequences. In this case, oligonucleotides consisting of or comprising sequences as set forth in SEQ ID NO. 12 to SEQ ID NO. 51 are particularly preferred.

The invention likewise relates to a diagnostic test kit as described above, where the oligonucleotides made available in this kit are provided with a detectable marker. Such detectable markers can include, inter alia, enzymes, enzyme substrates, coenzymes, enzyme inhibitors, fluorescence markers, chromophores, luminescent markers and radioisotopes.

This invention likewise relates to antibodies which react specifically with an epitope of a ß-tubulin from nematodes of the family of the Strongylidae, preferably of the subfamily of the Cyathostominae, particularly preferably of the genera Cylicocyclus and Cyathostomum, very particularly preferably of the species *Cylicocyclus nassatus* and *Cyathostomum coronatum*.

This invention likewise particularly relates to monoclonal antibodies which react specifically with an epitope of a ß-tubulin from nematodes of the family of the Strongylidae, preferably of the subfamily of the Cyathostominae, particularly

preferably of the genera Cylicocyclus and Cyathostomum, very particularly preferably of the species *Cylicocyclus nassatus* and *Cyathostomum coronatum*.

This invention likewise relates to the use of the abovementioned antibodies as nematicides.

This invention also relates to the use of the abovementioned ß-tubulin polypeptides or fragments thereof from nematodes of the family of the Strongylidae, preferably of the subfamily of the Cyathostominae, particularly preferably of the genera Cylicocyclus and Cyathostomum, very particularly preferably of the species *Cylicocyclus nassatus* and *Cyathostomum coronatum*, for the preparation of vaccines which contain at least one of the ß-tubulin polypeptides mentioned or fragments thereof. The vaccine is in this case able to produce an immune response which is specific for a ß-tubulin described above.

In a preferred embodiment, the vaccine contains an antigenic determinant, e.g. an individual determinant of a polypeptide having an amino acid sequence as set forth in SEQ ID NO. 2, 4, 6, 8 or 10 or of a polypeptide which is encoded by one of the abovementioned DNAs or fragments thereof.

The invention likewise relates to a procedure for the preparation of an immunogenic composition for the immunization of mammals, consisting of at least one of the abovementioned ß-tubulin polypeptides according to the invention or fragments thereof or of at least one of the abovementioned antibodies.

The invention likewise relates to the use of the expression vectors described above containing a nucleic acid coding for a β-tubulin according to the invention, preferably a sequence as set forth in SEQ ID NO. 1, 3, 5, 7, 9 or 11, fragments thereof or sequences homologous thereto for the preparation of an immunogenic composition for administration to a host for the activation of a protective immune response in this host, which is directed at β-tubulin from parasitic nematodes.

The invention likewise relates to an immunogenic composition comprising a vector (comprising a nucleic acid coding for the β-tubulin according to the invention, preferably a sequence as set forth in SEQ ID NO. 1, 3, 5, 7, 9 or 11, fragments thereof or sequences homologous thereto and to a promoter sequence which is linked functionally to said nucleotide sequence and which controls the expression of the β-tubulin according to the invention producing an immune response) and a vehicle suitable for pharmaceutical purposes.

This invention likewise relates to a process for the identification of substances which modulate the interaction of tubulin or the interaction of subunits of tubulin. The process is based on the use of tubulin, preferably on tubulin from parasitic nematodes, particularly preferably on ß-tubulin from parasitic nematodes of the order strongylida, very particularly preferably on ß-tubulin from parasitic nematodes of the family of the Strongylidae, most preferably on ß-tubulin from parasitic nematodes of the subfamily of the Cyathostominae. A particularly preferred group of the ß-tubulin used in this procedure is ß-tubulin from parasitic nematodes of the genera Cylicocyclus and Cyathostomum.

The invention relates to the identification of substances, e.g. small organic molecules, which are able to modulate the interaction of tubulin protein molecules or its subunits with one another. Preferably, the invention relates to the identification of compounds which inhibit the interaction.

The invention likewise relates to a procedure as described above, which is based on

a) bringing the substance to be tested into contact with the tubulin, the conditions selected allowing the interaction of the tubulin molecules with one another and the binding of the test substance to tubulin,

b) detecting the binding which has taken place by determining the ability of the tubulin protein molecules to interact with one another and

c) comparing the ability of the tubulin protein molecules to interact with one another in the presence of a test substance to the ability to interact with one another in the absence of a test substance.

The invention likewise relates to a process for the identification of substances which modulate the ability of tubulin molecules to interact with one another. Particularly preferably, the invention in this case relates to a procedure which uses one of the polypeptides described above which are coded by the DNAs described above or fragments thereof, particularly by DNAs consisting of or comprising sequences as set forth in SEQ ID NO. 1, 3, 5, 7, 9 or 11, and by sequences which have an identity of 85% thereto, preferably of 95% and code for ß-tubulin which has an amino acid sequence as set forth in SEQ 2, 4, 6, 8 or 10.

The invention likewise relates to a procedure for the identification of substances which modulate the ability of tubulin to interact with one another as described above, the procedure used being based on detecting a modulation of the tubulin interaction in the presence of a test substance with the aid of a test system based on cells. A preferred embodiment of such a test system is the "two hybrid system" (US 5 283 317, Zervos et al. (1993) Cell 72, 223-232; WO 94/10300). This system is suitable for documenting or describing the interaction of two proteins by the interaction leading to a detectable signal. Such a system can also be adapted to test systems having high throughput numbers.

The invention likewise relates to a procedure for the identification of substances which modulate the ability of tubulin to interact with one another, the procedure used being based on detecting a modulation of the tubulin interaction in the presence of a test substance with the aid of a cell-free test system. A particularly preferred embodiment of such a test system is the "scintillation proximity assay" (SPA)

(EP 015 473). This test system is based on the detection of an interaction of a receptor bound to microspheres or beads, e.g. of a tubulin molecule with a ligand, the microspheres or beads being provided with a scintillating molecule. A signal is detected if the receptor-ligand complex decomposes.

The invention likewise relates to substances which have hitherto still not been described, which are identified with the aid of the procedure described above and are suitable to modulate, preferably to inhibit, the interaction of tubulin molecules.

The invention likewise relates to the use of substances which have hitherto still not been described, which were identified using one of the procedures described above, for the preparation of an agent which is used for the prophylactic or therapeutic treatment of animals or humans which can be attacked or have been attacked by nematodes. The agents according to the invention contain at least one of the substances identified by one of the procedures described above and can be administered nasally, dermally, parenterally or enterally.

For better understanding, the meaning of certain words and terms which are used in the description, the examples and attached claims are explained in greater detail below.

The term "fragments" with respect to proteins and DNA describes parts of the nucleic acids or amino acid sequences described under SEQ ID NO. 1 to 11, sequences complementary thereto or sequences identical thereto to 85%, preferably to 95%. The fragments of the DNA and polypeptide sequences comprise at least five nucleotides or amino acids, but can likewise comprise up to 447 amino acids or up to 1343 nucleotides, or up to 2565 nucleotides in the case of the sequence as set forth in SEQ ID NO. 11.

The terms "homology", "identity" or "similarity" relate to sequence similarities between two peptides or between two nucleic acid molecules. Homology can be

determined by in each case comparing a position in each sequence with one another. If a position in the compared sequence is occupied by the same base or amino acid, the two molecules in this position are homologous. The measure of homology between sequences is a function of the number of the corresponding or homologous positions which the sequences share with one another. A "nonhomologous" sequence has an identity of less than 40%, but preferably of less than 25% identity.

The term "homology" in particular means that DNA segments of a length of at least 15 base pairs or strands complementary to the DNA agree to at least 85%, preferably 95%, with the nucleotides with the corresponding DNA. A homology can be determined, inter alia, with the aid of computer programs such as the GCG program (Devereux et al. (1983), Nucleic Acids Res. 12, 387-395).

A "homology" also exists if a DNA segment can hybridize to the DNA strand concerned or its complementary strand.

The term "hybridize" or "hybridization" describes the process in which a single-stranded nucleic acid molecule undergoes base pairing with a complementary DNA strand, the ability of a single-stranded nucleic acid molecule depending on the stringency of the hybridization conditions.

The term "stringency" relates to the hybridization conditions. "High stringency" is imparted if base pairing is rendered difficult. "Low stringency" is imparted if base pairing is facilitated.

The term "complementary" relates to the ability of purine and pyrimidine nucleotides to form base pairs with one another via hydrogen bridges. Complementary base pairs are, inter alia, guanine and cytosine, adenine and thymine and adenine and uracil.

The person skilled in the art realizes that, on account of the degenerate genetic code (i.e. 64 codons code for 20 amino acids), numerous "silent" substitutions of

nucleotide base pairs can be introduced into the sequence coded therefor without changing the identity of the protein products encoded thereby. All such substitutions are to be contained in the scope of the invention.

The term "specifically hybridize" relates to the ability of a nucleic acid molecule of the present invention to hybridize to at least approximately 6, 12, 20, 30, 50, 100, 150, 200, 300, 350, 400 or 440 successive nucleotides of one of the ß-tubulin genes described above, preferably to one of the sequences as set forth in SEQ ID NO. 1, 3, 5, 7, 9 or 11 or sequences homologous or complementary thereto, namely in such a way that ten times more molecules hybridize, preferably 100 times more molecules hybridize, and particularly preferably more than 100 times more molecules hybridize than to a cellular nucleic acid (e.g. mRNA or genomic DNA), which codes for a protein other than the ß-tubulin described above.

The term "plasmid" relates to an extrachromosomal genetic element. The original plasmids used for the present invention are either commercially obtainable, freely accessible or can be derived from such plasmids according to known processes.

The term "vector" describes a DNA element which is used for the introduction of exogenous DNA into host cells. A vector contains a nucleotide sequence which codes for one or more polypeptides. Vectors which are able to control the expression of the genes which they contain are designated as "expression vectors".

The term "gene" relates in the scope of the present invention to a nucleic acid comprising an open reading frame which codes for one of the ß-tubulin polypeptides described above. Exon and possible intron sequences are additionally included here.

The term "interact" or "interaction" describes detectable interactions between molecules. The term "binding" is additionally included here.

The term "modulate" relates both to a stimulation and to a suppression or inhibition of a biochemical process. In the context of the present invention, "modulation" means an inhibition or suppression of the interaction between tubulin polypeptides or fragments or subunits thereof, or a stimulation of this interaction which can be shown, for example, in an irreversible binding of tubulin polypeptides to one another.

The term "nucleic acid" relates to polynucleotides such as deoxyribonucleic acids (DNA) or, if appropriate, to ribonucleic acids (RNA). In an equivalent manner, the term also includes analogs of RNA or DNA which are prepared from nucleotide analogs, and, in the case concerned, single-stranded ("sense" or "antisense") and double-stranded polynucleotides.

The term "promoter" relates to DNA sequences which regulate the expression of a specific DNA, which are functionally linked to the promoter. The term also includes "tissue-specific" promoters, i.e. promoters which control the expression of the specific DNA only in certain cells (e.g. cells of a certain tissue). Likewise included are "tissue-nonspecific" promoters and promoters which lead to a constitutive expression or are inducible.

The terms "protein", "polypeptide" and "peptide" are exchangeable in their use in the context of the present application if they relate to a gene product.

A "fusion protein" is a fusion of a first amino acid sequence coding for one of the β-tubulin polypeptides described above having a second amino acid sequence which has no commonality or fundamental homology to the tubulin sequence. The second amino acid sequence can in this case originate from the same organism as the first, or alternatively can originate from another organism (intergenic). In general, a fusion protein can be represented by means of the formula X-tubulin-Y, where "tubulin" represents one of the polypeptides described above, and X and Y represent a polypeptide which is not connected to a tubulin amino acid sequence. X or Y can in each case independently of one another be absent.

The terms "cell" or "host cell" can be used in the same sense in the context of the application present here. It is understood that these terms relate not only to an individual cell, but also to the descendants of such a cell. On account of certain modifications in the course of following generations (e.g. mutations), such descendants are possibly not identical to the stem cell, but are additionally included by the present invention.

The term "intron" describes those sequences of the described, preferably genomic, DNA which are transcribed, but are then removed from the transcript by "splicing", the adjacent sequences (exons) being linked.

Nucleic acids

As already described, one aspect of the invention relates to nucleic acids from nematodes of the family of the Strongylidae, particularly of the subfamily of the Cyathostominae, very particularly to the genera Cyathostomum and Cylicocyclus, especially of the species *Cylicocyclus nassatus* and *Cyathostomum coronatum*, which code for ß-tubulin polypeptides or fragments thereof or nucleic acids homologous thereto which are homologous to 85% to the [lacuna] in SEQ ID NO. 1, 3, 5, 7, 9 and 11, preferably to 95%, and code for a ß-tubulin according to one of the sequences as set forth in SEQ ID NO. 2, 4, 6, 8 or 10 or fragments thereof. SEQ ID NO. 3 represents the degenerated sequence of the nucleic acid from *Cylicocyclus nassatus* coding for β-tubulin, where "r" represents a purine (guanine or adenine), "y" represents a pyrimidine (thymine, or uracil or cytosine) and "w" represents an adenine or a thymine, or a uracil. SEQ ID NO. 3 thus includes a number of sequences which can be present in the organisms of the species *Cylicocyclus nassatus*. The sequences as set forth in SEQ ID NO. 5, 7 and 11 show three defined sequences coding for ß-tubulin, which are exemplary and preferred embodiments of DNA as set forth in SEQ ID NO. 3.

Likewise part of the invention are oligonucleotides which optionally code for ß-tubulin polypentides which comprise a length of at least 2, 5, 10, 25, 50, 100, 150, 200, 250, 300, 350 or 400 amino acids. Such oligonucleotides can be used as primers or antisense molecules (i.e. as noncoding nucleic acids) and comprise at least approximately 6, 12, 24, 30, 60, 100, 120, 150 or 210 base pairs, while coding nucleic acids comprise approximately 200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200 or 1300 base pairs.

The invention also describes those oligonucleotides which specifically hybridize under stringent conditions to nucleic acids which are represented by one of the sequences as set forth in SEQ ID NO. 1, 3, 5, 7, 9 or 11. Correspondingly stringent conditions are, for example, 6 x sodium chloride/sodium citrate (SSC) at approximately 45°C, followed by a washing step with 2 x SSC at 50°C, and are familiar to the person skilled in the art (see, for example, Current protocols in Molecular Biology, John Wiley & Sons, N.Y. (1989), 6.3.1-6.3.6). Thus the salt concentrations in the washing step can be chosen such that the stringency is lower (2 x SSC, 50°C) or is higher (0.2 x SSC, 50°C). Furthermore, the temperature in the washing step can be varied, from conditions for a low stringency (e.g. about 22°C), up to conditions of higher stringency (e.g. about 65°C). Both salt concentration and temperature can be varied and tailored to one another.

Particularly preferred oligonucleotides which can be used as primers or for hybridization for the identification and characterization of an existing, e.g. genomic, DNA, are described in SEQ ID NO. 12-51. However, other oligonucleotides can also be derived from the sequences as set forth in SEQ ID NO. 1, 3, 5, 7, 9 or 11, which can then be used as primers or for hybridization. Particularly preferred for the identification of the species or genus to which an existing DNA can be assigned are those oligonucleotides which hybridize in the region of the intron of an existing (genomic) DNA. The introns of a genomic DNA coding for β-tubulin, which can be isolated from the abovementioned nematodes, are described by way of example of *Cylicocyclus nassatus* in SEQ ID NO. 11. The introns are thus those sequences which

are located between the coding exons. In a preferred embodiment, the described primers or hybridization probes contain a labeled group which makes possible the detection of the oligonucleotides, i.e. for example, radioisotopes, fluorescent groups, enzymes or enzyme cofactors.

Such oligonucleotides can be employed in diagnostic test kits in order to determine the origin (i.e. the organism) of an existing DNA. The oligonucleotides are suitable due to their specific hybridization with the DNAs mentioned under SEQ ID NO. 1, 3, 5, 7, 9 and 11 [lacuna] their fragments, sequences homologous thereto and sequences complementary thereto for recognizing defined sequences. Thus they also make possible the recognition and identification of those sequences coding for ß-tubulin which, on account of one or more point mutations in codon 200, lead to the expression of benzimidazole-resistant ß-tubulin. These oligonucleotides derived from the sequences SEQ ID NO. 1, 3, 5, 7, 9 and 11, preferably the embodiments described under SEQ ID NO. 12 to 51, are thus suitable for the identification of the frequently occurring nematode species of the subfamily of the cyathostominae, and for the recognition of existing resistances to benzimidazoles.

The oligonucleotides according to the present invention can be prepared using standard methods which are familiar to the person skilled in the art, e.g. by de novo DNA synthesis.

The nucleic acids mentioned here can be present in partially purified or biologically pure form in complete cells or in cell lysates, i.e. if other cell components or chemical precursors and by-products have been separated off in the case of a chemical synthesis of the DNA.

Nucleic acids coding for ß-tubulin, as described above, can be obtained starting from mRNA which is present in a number of eukaryotic cells. It is also possible to obtain the DNA according to the invention starting from genomic DNA from the nematode cells concerned (see also the following examples). A gene coding for ß-tubulin can

be obtained, for example, from a cDNA library or a genomic DNA library. cDNA can be obtained by isolating the total mRNA of a cell, e.g. of a nematode cell. Starting from the mRNA, double-stranded cDNA can then be prepared and inserted into a suitable plasmid or a suitable vector. The DNA according to the invention can also be obtained by amplification with the aid of the known polymerase chain reaction (PCR) or alternatively by de novo DNA synthesis (see also J. Sambrook et al. (1989) Molecular Cloning, 2nd Edition, Chap. 14).

Vectors and plasmids

The present invention also comprises expression vectors which contain one of the nucleic acid sequences according to the invention, which are functionally linked to a transcription-regulatory sequence. "Functionally linked" means that the nucleic acid sequence is linked to the regulatory sequence in a manner such that the expression of the protein encoded by the nucleic acid sequence can be controlled. "Transcription-regulatory sequences" include, for example, promoters, enhancers and other control elements. The expression vectors contain, for example, a gene coding for a ß-tubulin according to the invention or fragments thereof. These vectors can be used for incorporation into cells where the corresponding polypeptides or alternatively fusion proteins are then formed. Suitable promoters for the expression of the protein according to the invention in *E.coli* include natural hybrid or bacteriophage promoters. Preferably, they are promoters from the group of the phage λ promoters, omp promoters or synthetic promoters (see also WO 98/15625, DE 3 430 683, EP 0 173 149). Suitable vectors are commercially obtainable, e.g. the expression vectors of the pET series (e.g. pET3a, pET23a, pET28a and His tag or pET32a with His tag) or pGEX with glutathion synthetase fusion. The expression vectors can then be transformed, for example, into DE3-lysogenic E.coli strains, e.g. BL21(DE3), HM S 174(DE3) or AD494(DE3).

Expression of the ß-tubulin polypeptides

The present invention also comprises cells which contain the nucleic acid sequences according to the invention (e.g. inserted into a vector or into the genome). These host cells can be prokaryotic or eukaryotic.

Suitable prokaryotic expression systems are, for example, bacterial systems such as *Streptomyces* spp., *Bacillus subtilis, Salmonella typhimurium, Serratia marcescens* and preferably *E.coli*.

A preferred eukaryotic expression system is the baculovirus system, particularly preferably that which allows post-translational modifications.

Other eukaryotic expression systems (e.g. yeast, insect cells) can likewise be used.

Polypeptides

The present invention likewise comprises ß-tubulin polypeptides which are encoded by the DNAs according to the invention, preferably the DNA sequences as set forth in SEQ ID NO. 1, 3, 5, 7, 9 and 11, by fragments thereof or by homologous DNA sequences as described above. Preferred embodiments of these ß-tubulin polypeptides are described in the sequences as set forth in SEQ ID NO. 2, 4, 6, 8 and 10. In a preferred embodiment, the described polypeptides are purified polypeptides which are free of contaminating proteins of those cells in which the polypeptides according to the invention have been produced.

The polypeptides described are proteins of full length or fragments, motifs or domains thereof which comprise lengths of at least 5, 10, 25, 50, 75, 100, 125, 150, 200, 250, 300, 350 or 400 amino acids. Polypeptide fragments can be obtained and

are selected by the testing of polypeptides which are encoded by nucleic acid fragments derived from the sequences as set forth in SEQ ID NO. 1, 3, 5, 7, 9 and 11.

Polypeptide fragments can also be chemically synthesized in a known manner.

The invention also comprises polypeptides which are encoded by the degenerate sequence as set forth in SEQ ID NO. 3. Owing to the various possible bases in defined positions of the DNA sequence, various polypeptides result having an amino acid encoded by the codon resulting in each case. The polypeptides encoded by DNA as set forth in SEQ ID NO. 3 are described in SEQ ID NO. 4, the variable amino acids being marked by "Xaa".

Preferred embodiments of the polypeptides are described in SEQ ID NO. 2, 4, 6, 8 and 10.

The present invention comprises all processes for the preparation of the polypeptides according to the invention.

It is known to the person skilled in the art that the polypeptides of the present invention can be obtained in various ways, e.g. by chemical methods such as the solid-phase method. For the obtainment of larger amounts of protein, the use of recombinant methods is recommended.

The underlying steps for the preparation of the recombinant ß-tubulin are:

1. Obtainment of a natural, synthetic or semisynthetic DNA which codes for the ß-tubulin according to the invention.

2. Incorporation of this DNA into an expression vector which is suitable for expressing the ß-tubulin according to the invention, either on its own or as a fusion protein.

3. Transformation of a suitable, preferably prokaryotic, host cell using this expression vector.

4. Growth of this transformed host cell in a manner which is suitable for expressing the ß-tubulin according to the invention.

5. Harvesting of the cells and purification of the ß-tubulin by means of suitable, known methods.

For example, the expression vectors can be transformed in λDE3-lysogenic *E. coli* strains, e.g. BL21(DE3), HM S174(DE3) or AD494(DE3). After the growth of the cells under the standard conditions familiar to the person skilled in the art, expression is induced using IPTG. After induction of the cells, incubation is carried out at temperatures of 18 to 37°C for 3 to 24 hours. The cells are disrupted, the expressed protein is purified by means of chromatographic methods, in the case of protein expressed with His tag by means of FPLC on an Ni-NTA column, and also by ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis or alternatively immunoaffinity purification, which are specific for the polypeptides according to the invention.

Homologs or fragments of the polypeptides according to the invention can be generated by mutagenesis, such as, for example, by directed (point) mutagenesis, or by deletions.

The polypeptides according to the invention can also be chemically modified, e.g. with glycosyl groups, lipids, phosphates, acetyl groups or similar groups. Covalent derivatives can be obtained by the linkage of the modifying group with functional groups of the amino acid side chains or the N terminus or C terminus of the polypeptide.

In the expression of the polypeptides according to the present invention, it may be advantageous to change certain codons in order to make possible optimum expression. This is true if the use of certain codons ("codon usage") in the heterologous expression system is different than in one of the organisms according to the invention. Furthermore, the deletion of the 5'- or 3'-untranslated region is possible, e.g. if several destabilizing sequence motifs (e.g. ATTTA) are present in the 3' region of the cDNA.

Fusion proteins

The polypeptides according to the invention can also be present as part of a fusion protein. Such fusion proteins are embraced in full by the present invention. Fusion proteins can be useful under conditions where it is desirable to obtain an immunogenic fragment of the ß-tubulin (see, for example, EP 0 259 149; Schlienger et al. (1992) J. Virol. 66, 2). Under certain circumstances, fusion proteins facilitate the expression of a polypeptide. For example, the polypeptides according to the invention can be prepared as glutathione S-transferase (GST) fusion proteins. Such GST fusion proteins facilitate easy purification of the polypeptide (see, for example, Current Protocols in Molecular Biology, eds. Ausubel et al. (John Wiley & Sons, N.Y. 1991). Fusion proteins can contain, for example, a "leader" sequence which is used for the purification, e.g. allows a poly-His sequence at the N terminus (but also at the C terminus) of the protein whose purification [lacuna] by means of chromatography on an Ni^{2+} NTA column (see, for example, Hachuli et al. (1987) J. Chromatography 411, 177).

Techniques for preparing such fusion proteins are familiar to the person skilled in the art.

Antibodies

Another aspect of the present invention relates to antibodies which react specifically with the ß-tubulin polypeptides according to the invention.

For example, anti-protein or anti-peptide antisera or monoclonal antibodies can be prepared according to standard protocols by the use of immunogens which have been derived from β-tubulin polypeptides according to the invention (see, for example, Antibodies: A. Laboratory Manual ed. by Harlow and Lane (Cold Spring Harbor Press, 1988)).

Mammals such as mice, hamsters or rabbits can be immunized using an immunogenic form or an immunogenic fraction of the polypeptide according to the invention, e.g. using a polypeptide which is able to produce an antibody response (see also "fusion proteins" above). The appropriate techniques are familiar to the person skilled in the art. Thus an immunogenic fraction of the ß-tubulin can be administered in the presence of an adjuvant. The course of the immunization can be observed by checking the antibody titer in the plasma or serum, e.g. by customary ELISA assays or other immunoassays.

In a preferred embodiment, the antibodies according to the invention are immunospecific for an antigenic determinant of a ß-tubulin polypeptide according to the invention, e.g. of a polypeptide as set forth in SEQ ID NO. 2, 4, 6, 8 or 10 or those polypeptides which are encoded by DNAs as set forth in SEQ ID NO. 1, 3, 5, 7, 9 or 11 or sequences identical to 85% therewith, preferably sequences identical to 95%.

After the immunization of a mammal, polyclonal anti-ß-tubulin antibodies can be isolated from the serum. For the production of monoclonal antibodies, antibody-producing cells (lymphocytes) can be obtained from an immunized animal and fused

according to known methods with immortal cells such as myeloma cells in order to obtain hybridoma cells (see, for example, Köhler and Milstein (1975) Nature 256, 495-497; Kozbar et al. (1983) Immunology Today 4, 72; Cole et al. (1985) Monoclonal Antibodies and Cancer Therapy, Alan R. Liss, Inc. pp. 77-96).

The "antibodies" mentioned here should also include fragments of antibodies which react specifically with ß-tubulin according to the invention. Antibodies can be fragmented using conventional techniques and the fragments tested.

A preferred embodiment relates to antibodies such as described above, which carry a detectable label (e.g. radioisotopes, fluorescent groups, enzymes or enzyme cofactors).

Antibodies which bind specifically to ß-tubulin polypeptides according to the invention can also be used for the immunohistochemical staining of tissue samples in order to detect the expression of a specific β-tubulin. The anti-ß-tubulin antibodies can likewise be used for diagnostic purposes, e.g. for immunoprecipitation or for immunoblotting.

Diagnostic test procedures

The present invention likewise makes available nucleic acid molecules which can be used for diagnostic purposes.

These include nucleic acid molecules such as described above, the fragments of the DNA sequences described under SEQ ID NO. 1, 3, 5, 7, 9 or 11 or DNA sequences complementary thereto. For example, oligonucleotides as set forth in SEQ ID NO. 12 to 51 are made available, which are able to hybridize to sense or antisense sequences coding for ß-tubulin, and to intron sequence sections which are described by way of example in SEQ ID NO. 11.

In this procedure, the nucleic acid of a cell is rendered accessible to hybridization, the DNA probe is brought into contact with the oligonucleotides, and the hybridization of the sample is detected using the oligonucleotide.

In this manner, a method is made available which makes it possible to differentiate between various species of small strongylids and/or other nematode species by means of the specific hybridization of the oligonucleotides according to the invention to a DNA probe, preferably with the aid of those oligonucleotides which hybridize to the intron regions of the DNA coding for ß-tubulin. In a particularly preferred embodiment, the oligonucleotides according to the invention allow the identification of resistances in small (Cyathostaminae) e.g. in horses, especially resistances of the species *Cylicocyclus nassatus*, *Cyathostomum coronatum* and *Cyathostomum catinatum*. In this procedure, the fact can be used that resistant forms of the ß-tubulin carry at least one point mutation in the DNA according to the invention coding therefor, which can be detected by means of PCR, such as described, for example, in an analogous manner in Elard et al. (1998) PCR diagnosis of benzimidazole-susceptibility or –resistance in natural populations of the small ruminant parasite, *Teladorsagia circumcincta*. Veterinary Parasitology 80, 231-237.

The method described is particularly helpful for the assessment of possible treatment strategies in humans and animals affected with nematodes, such as, for example, horses, sheep, pigs, goats, camels, buffalo, donkeys, hares, roe deer, fur-bearing animals, birds (e.g. chickens, turkeys, ducks), fresh- and salt-water fish (e.g. trout, carp). It makes possible the identification and differentiation of the parasitic nematodes and the recognition of resistant populations thereof, and avoid a treatment with inactive nematicides.

The methods described here can be made available, for example, in the form of prefabricated diagnostic test kits which contain at least one of the abovementioned nucleic acid molecules or an antibody such as described above, which is prepared in ready-to-use form.

Procedure for the discovery of nematicidal substances

The invention relates to a procedure in which, with the aid of tubulin or fragments thereof, novel, specific anthelmintic substances can be identified.

In a preferred embodiment, β-tubulin polypeptides according to the present invention are used for this. However, the procedure can also be carried out using tubulin from species other than those mentioned here. Procedures which use β-tubulin polypeptides other than those according to the invention are included in full by the present invention. Particularly preferably, β-tubulin polypeptides as set forth in SEQ ID NO. 2, 4, 6, 8 or 10 are used for the procedure mentioned. In the present invention, recombinant β-tubulin polypeptides from frequently occurring parasitic nematodes are thus additionally made available. These can be used in various test systems for the identification of new inhibitors of tubulin interaction, or the interaction of tubulin subunits.

Cell-free test systems

Many test systems which have the testing of compounds and natural extracts as their aim are aimed at high throughput numbers in order to maximize the number of substances investigated in a given period of time. Test systems which are based on cell-free studies use purified or semi-purified protein. They are suitable for a "first" test, which primarily aims at detecting a possible influence of a substance on the target protein.

Effects such as cell toxicity are as a rule ignored in these *in vitro* systems. The test systems in this case check both inhibitory and suppressive effects of the substances, and stimulatory effects. The effectiveness of a substance can be checked by means of concentration-dependent test series. Control batches without test substances can be used for the assessment of the effects.

One possibility for the identification of substances which modulate the interaction of tubulin or of its subunits is the "scintillation proximity assay" (SPA), see EP 015 473. This test system uses the interaction of a receptor (e.g. tubulin) with a radiolabeled ligand (e.g. a small organic molecule or a second, radiolabeled protein molecule). The receptor is in this case bound to small spheres ("microspheres") or beads which are provided with scintillating molecules. In the course of the decay of the radioactivity, the scintillating substance in the microsphere is excited by the subatomic particles of the radioactive label and a detectable photon is emitted. The test conditions are optimized such that only those particles emanating from the ligand lead to a signal which emanate from a ligand bound to the receptor or the tubulin.

In one possible embodiment, tubulin is bound to the beads, either together or without interacting or binding test substances. α- or β-Tubulin subunits could be used here. A radiolabeled ligand could be, for example, a labeled benzimidazole or a further, labeled β-tubulin molecule. In the case of binding of the ligand to the immobilized tubulin, this ligand must inhibit or abolish an existing interaction between immobilized and free tubulin in order to bind itself in the region of the contact surface. Binding to the immobilized tubulin which has taken place can then be detected by means of a flash of light. Correspondingly, an existing complex between an immobilized and a free, labeled tubulin is destroyed by the binding of a test substance, which leads to a decrease in the detected intensity of the flash of light. The test system then corresponds to a complementary inhibition system.

Test system based on cells

The β-tubulin available by means of the present invention, but also tubulin from other species, makes possible the development of test systems, which are based on cells, for the identification of substances which inhibit the tubulin interaction.

An example of such a test system is the "two hybrid system". A specific example of this is the "interaction trap". This is a genetic selection of interacting proteins in yeast (see, for example, Gyuris et al. (1993) Cdi 1, a human G1 and S phase protein phosphatase that associates with Cdk 2. Cell 75, 791-803). The test system is designed to detect and to describe the interaction of two proteins in that an interaction which has taken place leads to a detectable signal.

Such a test system can also be adapted to the testing of large numbers of test substances in a given period of time.

The system is based on the construction of two vectors, the "bait" vector and the "prey" vector. A gene coding for tubulin, preferably a gene coding for a β-tubulin according to the invention, is cloned in the bait vector and then expressed as a fusion protein with the LexA protein, a DNA-binding protein. A second gene, coding for tubulin, preferably for a β-tubulin according to the invention, is cloned in the prey vector, where it is expressed as a fusion protein with the B42 prey protein. Both vectors are present in a *Saccharomyces cerevisiae* host, which contains copies of a LexA-binding DNA on the 5'-side of a lacZ or HIS3 reporter gene. If an interaction takes place between the two tubulin (fusion) proteins, activation of the transcription of the reporter gene occurs. If the presence of a test substance leads to the inhibition or disturbance of the tubulin interaction, the two tubulin (fusion) proteins can no longer interact and the product of the reporter gene is no longer prepared.

With the aid of tubulin, particularly of the β-tubulin according to the invention or fragments thereof, and the processes described above, it is possible to identify new and specific antiparasitic compounds.

Compounds which are found with the aid of the processes and polypeptides described are valuable for the treatment of humans and animals which are infected with pathogenic endoparasites of the human or of agricultural animals, pets, zoo animals, and laboratory and experimental animals.

The compounds are active against all stages of development of normal, sensitive strains and also resistant strains. By treatment with agents which contain one or more of these compounds, both economic losses in the case of agricultural animals and diseases in humans and animals can be avoided or treated. The following parasites are in this case of particular interest as targets of the active compounds found:

Enoplida, e.g. *Trichuris* spp., *Capillaria* spp., *Trichomosoides* spp., *Trichinella* spp.

Rhabditia, e.g. *Micronema* spp., *Strongyloides* spp.

Strongylida, e.g. *Strongylus* spp., *Triodontophorus* spp., *Oesophagodontus* spp., *Trichonema* spp., *Gyalocephalus* spp., *Cylindropharynx* spp., *Poteriostomum* spp., *Cyclococercus* spp., *Cylicostephanus* spp., *Oesophagostomum* spp., *Chabertia* spp., *Stephanurus* spp., *Ancylostoma* spp., *Uncinaria* spp., *Bunostomum* spp., *Globocephalus* spp., *Syngamus* spp., *Cyathostomum* spp., *Cylicocyclus* spp., *Neostrongylus* spp., *Cystocaulus* spp., *Pneumostrongylus* spp., *Spicocaulus* spp., *Elaphostrongylus* spp., *Parelaphostrongylus* spp., *Crenosoma* spp., *Paracrenosoma* spp., *Angiostrongylus* spp., *Aelurostrongylus* spp., *Filaroides* spp., *Parafilaroides* spp., *Trichostrongylus* spp., *Haemonchus* spp., *Ostertagia* spp., *Marshallagia* spp., *Cooperia* spp., *Nematodirus* spp., *Hyostrongylus* spp., *Obeliscoides* spp., *Amidostomum* spp., *Ollulanus* spp.

Oxyurida, e.g. *Oxyuris* spp., *Enterobius* spp., *Passalurus* spp., *Syphacia* spp., *Aspiculuris* spp., *Heterakis* spp.

Ascaridia, e.g. *Ascaris* spp., *Toxascaris* spp., *Toxocara* spp., Parascaris spp., *Anisakis* spp., *Ascaridia* spp.

Spirurida, e.g. *Gnathostoma* spp., *Physaloptera* spp., *Thelazia* spp., *Gongylonema* spp., *Habronema* spp., Parabronema spp., *Draschia* spp., *Dracunculus* spp.

Filariida, e.g. *Stephanofilaria* spp., *Parafilaria* spp., *Setaria* spp., *Loa* spp., *Dirofilaria* spp., *Litomosoides* spp., *Brugia* spp., *Wuchereria* spp., *Onchocerca* spp.

Gigantorhynchida, e.g. *Filicollis* spp., *Moniliformis* spp., *Macracanthorhynchus* spp., *Prosthenorchis* spp.

Mastigophora (Flagellata)

Trypanosomatidae, e.g. *Trypanosoma b. brucei, T. b. gambiense, T. b. rhodesiense, T. congolense, T. cruzi, T. evansi, T. equinum, T. lewisi, T. percae, T. simiae, T. vivax, Leishmania brasiliensis, L. donovani, L. tropica*

Trichomonadidae , e.g. *Giardia lambilia, G. canis.*

Sarcomastigophora (Rhizopoda), e.g. *Entamoeba histolytica*

Hartmanellidae, e.g. *Acanthamoeba* sp., *Hartmanella* spp.

Apicomplexa (Sporozoa), e.g. *Eimeria acervulina, E. adenoides, E. alabahmensis, E. anatis, E. anseris, E. arloingi, E. ashata, E. auburnensis, E. bovis, E. brunetti, E. canis, E. chinchillae, E. clupearum, E. columbae, E. contorta, E. crandalis, E. deblieckí, E. dispersa, E. ellipsoidales, E. falciformis, E. faurei, E. labbeana, E. leucarti, E. magna, E. maxima, E. media, E. meleagridis. E. meleagrimitis, E. mitis, E. necatrix, E. ninakohlyakimovae, E. ovis, E. parva, E. pavonis, E. perforans, E. phasani, E. piriformis, E. praecox, E. residua, E. scabra, E.* spec., *E. stiedai, E. suis, E. tenella, E. truncata, E. truttae, E. zuernii, Globidium* spec., *Isospora belli, I. canis, I. felis, I. ohioensis, I. rivolta, I.* spec., *I. suis, Neospora caninum, Cystisospora* spec. *Cryptosporidium* spec.

Toxoplasmadidae, e.g. *Toxoplasma gondii*

Sarcocystidae, e.g. *Sarcocystis bovicanis, S. bovihominis, S. neuvona, S. ovicanis, S. ovifelis, S.* spec., *S. suihominis*

Leucozoide, e.g. *Leucozytozoon simondi*

Plasmodiidae, e.g. *Plasmodium berghei, P. falciparum, P. malariae, P. ovale, P. vivax, P.* spec.

Piroplasmea, e.g. *Babesia argentina, B. bovis, B. canis, B.* spec., *Theileria parva, T.* spec.

Adeleina, e.g. *Hepatozoon canis, H.* spec.

The following are furthermore of importance

Myxospora and Microspora, e.g. *Glugea* spec. and *Nosema* spec., and *Pneumocystis carinii*, Ciliophora (Ciliata), e.g. *Balantidium coli, Ichthiophthirius* spec., *Trichondina* spec. or *Epistylis* spec.

The compounds and agents found are likewise effective against protozoa of insects, such as those of the strain Microsporidia, particularly those of the order Nosema, very particularly those of the species Nosema apis, which are parasites of honeybees.

Examples

Example 1

Obtainment of β-tubulin cDNA and genomic DNA

mRNA was obtained with the aid of the Quick Prep® Micro mRNA kit (Pharmacia Biotech, Freiburg, Germany) from *C. nassatus* worms and from *C. coronatum* and *C.catinatum* worms using the Dynal® mRNA Direct Kit (Dynal, Hamburg, Germany). The worms were isolated from the large intestine of horses and differentiated microscopically according to the characterized structure of head and tail (see R. S. Lichtenfeld (1975), Helminths of domestic equids. Proceedings of the Helminthological Society, Washington, 42 (Special issue), 1-92).

The synthesis of the cDNA was carried out with the aid of the "reverse transcription system" (Promega, Madison, USA) in the case of the mRNA from *C. nassatus* and using the superscript RTII reverse transcriptase (Gibco BRL Life Technologies) in the case of the mRNA from *C. coronatum* and *C. catinatum*. In the cases mentioned, oligonucleotides having a length of 15 base pairs were used. Incubation was carried out for one hour at 42°C.

Genomic DNA was obtained from 4 to 40 adult worms using the QIA Amp-tissue kit (Qiagen, Hilden, Germany). In this procedure, the worms were digested for 2 hours at 55°C with proteinase K and the genomic DNA extracted using "spin columns".

Example 2

Amplification of β-tubulin sequences

The amplification of β-tubulin sequences of full length or of fragments can be carried out, for example, using AmpliTaq Gold™ polymerase (Perkin Elmer, Foster City, California, USA).

An amplification of the β-tubulin sequences as set forth in SEQ ID NO. 1, 3, 5, 7, 9 or 11 or fragments thereof can be carried out with the aid of the primers as set forth in SEQ ID NO. 12 - 51.

For the amplification of the cDNA from *C. coronatum*, for example, the sequences as set forth in SEQ ID NO. 43 and 44 are suitable, for the amplification of the cDNA from *C. catinatum*, the sequences as set forth in SEQ ID NO. 40 and 42 are particularly suitable.

The amplification of the *C. nassatus* cDNA and of the genomic DNA of all species according to the present invention was carried out in a total volume of 50 µl, containing 5 µl of 10 x buffer, 2.5 µl of $MgCl_2$ (25 mM), 2 µl of dNTP mix (2mM for each NTP), 1 µl of each specific primer (SEQ ID NO. 12 - 47) (50 p mol/µl), 0.5 µl (2.5 U) of polymerase and 1 - 5 µl of DNA template. When using degenerate primers (SEQ ID NO. 48 - 51), 1 µl of each primer of a conc. of 500 pmol/µl was employed. The annealing was carried out in the case of degenerate primers at 46°C, in the case of specific primers the temperature was varied according to the calculated melting temperature. The PCR cycles were chosen as follows:

95°C for 10 min, then 35 - 40 cycles with 1 min denaturation at 94°C, 1 min annealing, 1 min at 72°C and a final step at 72°C for 10 min. In the amplification of cDNA from *C. coronatum* and *C. catinatum*, a "touchdown" PCR temperature program was carried out, which has the following profile:

firstly 15 cycles at 94°C for 30 sec, then 1 min at 60°C and 1 min at 72°C, followed by 15 cycles with 30 sec at 95°C, 55°C for 1 min and 72°C for 1 min and finally 10 cycles at 95°C for 30 sec, then 45°C for 1 min and 72°C for 1 min. For the amplification of larger fragments (>1000 base pairs), the elongation phase at 72°C was lengthened to 2.30 min.

Example 3

PCR products from the amplification of cDNA or genomic DNA from *C. nassatus, C. coronatum* and *C. catinatum* were cloned with the aid of the "Original TA Cloning Kit" /Invitrogen, Leek, Netherlands), namely in the "Original TA Cloning®" vector.